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(54) Title: **ANTIVIRAL OR ANTIFUNGAL COMPOSITION AND METHOD**

(57) Abstract

Antiviral and antifungal compositions comprise a mixture of a ferrous salt and a plant extract of pomegranate rind, *Viburnum plicatum* leaves or flowers, tea leaves or maple leaves in aqueous solution.

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ANTIVIRAL OR ANTIFUNGAL COMPOSITION AND METHOD

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Bacteriophage represent a diverse group of viruses that exert both positive and negative effects in microbiology. For example, in the dairy industry bacteriophages of the lactic acid bacteria represent a major source of starter culture failure with consequent poor-quality milk fermentations. A number of measures to prevent phage infection, including implementation of hygienic procedures, rotation of starter cultures, isolation of resistant mutants and formulation of media to suppress phage proliferation have been instituted with various degrees of success [1-4]. By contrast, coliphage have proven to be valuable monitors of faecal contamination in ground water and treated drinking water [5] and are effective viral models in studies of water quality and virucidal activity [6, 7]. Recent developments with genetically recombinant bacteriophage, containing either the bacterial luciferase *lux* genes or the ice nucleation gene *ina*, have established an additional value for phage in the rapid detection of bacteria in food and environmental samples [8-12]. In the above examples, the eventual destruction of bacteriophage is important either from the perspective of maintaining effective fermentation or from the viewpoint of good microbiological practice in the disposal of contaminated material. At present, the methods available for bacteriophage destruction such as heat and chemical disinfection [13, 14], have a significant impact on the viability and survival of associated bacteria. In the case of a sterilisation regime this is of little significance but the ability to combat bacteriophage in industrial environments

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would benefit from an environmentally benign procedure that could differentially destroy the virus without damaging metabolically-active bacterial cells. One example is in the dairy industry as mentioned above.
5 Another concerns spraying plants which have symbiotic bacteria to control bacteriophage infection.

There are also many situations where it would be useful to be able to combat fungus without at the same time damaging metabolically-active cells. Plant
10 extracts have been used against disease development in banana by fungi [15].

This invention provides an antiviral or antifungal composition comprising an effective concentration of a mixture of a ferrous salt and an
15 extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves.

An antiviral composition is one which combats, e.g. by preventing growth or preferably
20 killing, virus such as bacteriophage. An antifungal composition is one which combats, e.g. by preventing growth and preferably by killing fungus and destroying its spores. Preferred compositions are antiviral or
25 antifungal, but without at the same time substantially damaging metabolically active bacterial or other cells with which the virus or fungus is associated. Preferably the composition is an aqueous solution, i.e. one in which water is the sole or the main constituent solvent.

30 The composition comprises a ferrous salt, which may conveniently be ferrous sulphate. The nature of the anion is however not critical, provided that the salt is non-toxic to bacterial and other cells under the intended conditions of use and is water-soluble. A
35 preferred ferrous salt concentration range is 0.1 mM to 0.1 M, particularly from 1 to 20 mM.

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The composition also contains an extract of a plant. These plant extracts may conveniently be prepared by boiling the comminuted plant part with water or other solvent. The resulting extract may be
5 fractionated. It is probable that the extract contains one or more active components. Although such active components have remained refractive to purification, there is a uniform consistency in the extracted activity from different sources of the plant parts and
10 from parts obtained at different times of the year. The inventors have examined many different plants and have identified the following four as active:

- Pomegranate rind. Whole pomegranates can be comminuted and used, but the activity resides in the
15 rind.

- Viburnum plicatum leaves or flowers.

- Tea leaves. These may be dried or green. Other parts of the tea plant Camellia sinensis may be used.

20 - Maple leaves e.g. UK Acer pseudoplatanus or Canadian maple leaves, or more generally leaves or flowers of any part of the genus Acer.

The plant extract may be used as is, or diluted as appropriate, e.g. by a factor of up to 100.
25 Effective compositions generally contain 10-90% by volume of the ferrous salt solution together with correspondingly 90-10% by volume of the concentrated or diluted plant extract. The composition should preferably be stored in the dark.

30 The invention also includes solid or liquid concentrates which on dilution with water gives compositions as described.

The invention also includes a method of controlling virus or fungus, which method comprises
35 contacting the virus or fungus with an effective concentration of a ferrous salt and an effective

concentration of an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves. These two components can be used in sequence in either order. Preferably,
5 however they are used mixed together as a composition as described above.

The virus or fungus to be controlled may be contacted with, e.g. immersed in, the composition, typically for a few seconds or minutes. Where the
10 virus or fungus is on a surface, the composition may be applied to the surface, e.g. by spraying or wiping.

The surface may be for example a work surface or a vessel or utensil used in a hospital or kitchen or an industrial environment, or an external surface of a
15 mammal e.g. a human or a plant. Or a solution containing virus or fungus may be mixed with a composition as defined.

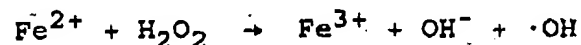
As described in the examples below, ferrous sulphate in combination with selected plant extracts
20 effects complete destruction of a broad range of bacteriophage infecting diverse bacterial genera. In assays incorporating both bacteriophage and bacteria at 10^{12} and 10^9 /ml respectively, the bacteriophage are entirely destroyed within two minutes without affecting
25 bacterial viability as measured by colony forming ability.

When used alone, ferrous sulphate has virucidal activity against phages, and also bactericidal activity against some bacteria. This
30 invention is based on the observation that ferrous sulphate, either alone or in combination with certain plant extracts, offers a potent broad spectrum virucidal activity. Since the activity of relatively low levels of ferrous sulphate can be further
35 potentiated by the addition of trace amounts of hydrogen peroxide (data not shown) it is likely that

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the mechanism of action involves, at least in part, a free radical system. A mechanism similar to that operating in the phagolysosome and defined by the Modified Haber-Weiss Reaction [17] is proposed.

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While resistance of bacterial cells may be effected through free radical scavenging and repair systems, it is clear that the plant extracts also have a role and that remains to be elucidated.

Materials and Method

Bacteria and bacteriophage strains

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One Gram-positive Staphylococcus aureus NCIMB 8588 and two Gram-negative Salmonella typhimurium LT2 and Pseudomonas aeruginosa NCIMB 10548 bacteria were used. The bacteriophage with specificity for the above bacteria were NCIMB 9563 for Staph. aureus, Felix 01 for S. typhimurium [16] (obtained from Amersham International plc., Amersham, HP7 9NA, UK) and NCIMB 10116 and 10884 for Ps. aeruginosa. (All these bacteria and bacteriophage are available to the public.)

20

The bacterial cells were maintained on Tryptose Phosphate Broth (TPB; Oxoid) supplemented with 1% agar (TPA) and stored at 4°C with monthly subculture. When required, cells were resuscitated in 10 ml TPB (18h, 37°C) or an orbital shaker operating at 240 rpm. Appropriate bacterial dilutions were made in Lambda buffer (6 mM Tris, 10 mM MgSO₄·7H₂O, 50 µg/ml gelatin; pH 7.2). After treatment bacterial survival was determined by colony forming units (cfu) on TPA [18].

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Phage stocks were developed on their appropriate host strains by a plate lysis procedure essentially equivalent to growing bacteriophage Lambda-derived vectors [19]. Typical phage titres of 10¹²/ml

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were obtained. Phage stocks were maintained in Lambda buffer at 4°C and stocks retained a constant titre for several months.

5 Preparation of plant extracts

Pomegranate rind, Viburnum plicatum leaves or flowers, maple leaves and commercial tea leaves were blended in distilled water (25% w/v) and boiled for 10 min. After centrifugation (20,000 x g, 4°C, 10 30 min), supernatants were autoclaved (121°C, 15 min), cooled and stored at -20°C. A further purification of the pomegranate extract to a molecular weight cut-off of 10,000 Da was achieved by membrane ultra filtration and the filtrate stored as above.

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Example 1

Preparation of Composition A

20 a. Preparation of 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in Lambda-buffer.

First freshly prepare stock solution (0.53%) of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.053 gm ferrous sulphate in 10 ml Lambda-buffer). After sterilisation by membrane filtration (0.45 μm , Whatman) prepare the final ferrous sulphate concentration of 4.3 mM by transferring 4.1 ml 25 of the ferrous stock solution to a sterile test tube containing 14 ml of Lambda-buffer.

b. Preparation of 13% PRE (pomegranate rind extract):

30 Mix 1.3 ml of stock solution of PRE (25% w/v) with 8.7 ml of Lambda-buffer.

Composition A was prepared 1-2 min prior to use by mixing 16.74 ml of 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (a; yellow) with 8.265 ml of 13% PRE (b; yellow). After about 30 sec the colour of the mixture (a and b) 35 changed greenish then to black. These mixtures of ferrous sulphate and PRE (a and b) should be protected

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from light.

Example 2

5 Virucidal Assay

Plant extracts were diluted 1:8 in Lambda buffer immediately prior to use and 300 μ l of this diluted extract added to 700 μ l of freshly prepared ferrous sulphate solution (4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; pH 6.5);
10 these mixtures should be protected from light. Bacteriophage (20 μ l at 10^{12} pfu/ml) or 20 μ l of an appropriate dilution of bacteria (10^9 cfu/ml) were placed in a sterile Eppendorf micro-centrifuge tube and 144 μ l of the FeSO_4 solution or PRE solution or
15 composition A (Example 1) or of the above plant extract/ FeSO_4 mixture added. After exposure of the bacteriophage or bacteria for 2 min at room temperature the activity of the mixture was neutralised by adding an equal volume of 2% (v/v) Tween 80 in Lambda buffer.
20 The number of bacteriophage or bacteria surviving the above protocol were measured by plaque forming units (pfu) or colony forming units respectively.

The results are set out in the following Tables 1 and 2. Table 1 indicates that pomegranate
25 rind extract alone has a slight virucidal activity against Pseudomonas phage but in combination with FeSO_4 there is a profound synergy. Eleven log reductions in plaque forming ability are obtained within 2 min. A similar synergy of virucidal action was achieved with
30 other plant extracts, leaves and flowers of Viburnum plicatum, maple leaves and tea leaves (Table 2).

Our particular interest in the above studies was to select agents with maximal virucidal activity whilst having little effect on bacteria. In this
35 regard FeSO_4 , either alone or in combination with pomegranate rind extract, appears singularly

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successful. Table 1 shows that for each bacterium and phage combination tested there is a simple treatment that can eliminate bacteriophage activity without affecting bacterial visibility. The plant extracts
5 appear to serve a dual role in their interaction with FeSO_4 . In the case of Pseudomonas phage they promote virucidal activity while for the Salmonella phage, which is completely inactivated by FeSO_4 alone, the pomegranate rind extract appears to provide a
10 significant protection to the bacterium.

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Table 1 The survival of different bacteria and bacteriophage species in 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 13% PRE (pomegranate rind extract; 20 min at 37°C) and Composition A (PRE + 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 2 min at room temperature) prepared in Lambda buffer.

Microorganisms	Lambda Buffer	13% PRE	4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Composition A*
Bacteriophage species (pfu/ml):				
<u>Staphylococcus</u> NCIMB 9563	7×10^{11}	2×10^7	Nil	Nil
<u>Salmonella</u> Felix D1	3×10^{12}	3×10^{12}	Nil	Nil
<u>Pseudomonas</u> NCIMB 10884	5×10^{11}	5×10^9	5×10^{11}	Nil
<u>Pseudomonas</u> NCIMB 10116	5×10^{11}	5×10^8	5×10^{11}	Nil
Bacteria species (cfu/ml):				
<u>Staph. aureus</u> NCIMB 8588	3×10^9	2×10^6	3×10^9	5×10^6
<u>S. typhimurium</u> LT2	2×10^9	2×10^9	Nil	2×10^9
<u>Pe. aeruginosa</u> NCIMB 10548	4×10^9	4×10^9	1×10^7	4×10^9

* Escherichia coli phage M13mp18 was killed by Composition A, whilst E. coli JM101 was unaffected.

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Table 2. Effect of 25% solutions of different plant extracts in the presence of 4.3 mMol ferrous sulphate on the inactivation within 3 minutes of Ps. aeruginosa bacteriophage NCIMB 10116.

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25% Plant extract solution (%v/v)	PFU/ml
Lambda Buffer (control)	1.2×10^{10}
Commercial tea leaves	Nil
<u>Viburnum plicatum</u> leaves or flowers	Nil
Maple leaves	Nil

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An antiserum was raised in rabbits against the Pseudomonas phage. A classic precipitin band was obtained with a double-immunodiffusion assay of phage antigen against this antiserum. No such precipitin band was obtained, however, when the phage had been treated with the virucidal agent, nor if the antiserum had been raised against treated phage.

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Treated phage were further examined by electron microscopy. No recognisable phage morphological structures remained in any of the twenty fields examined. The result of the further tests are consistent, therefore, with a complete destruction of the bacteriophage structure by the virucidal composition, to an extent that antigenic determinants are lost.

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Example 3Antifungal Test

5 Apple leaves infected with powdery mildew
were immersed in Composition A (Example 1) for about 60
seconds or until the leaves were completely wetted.
The leaves were then observed daily. The powdery
mildew was completely inhibited within 24 hours and no
mildew lesions were present on the treated apple leaves
10 as they hardened off.

A further microscopic test of treated powdery
mildew spores and mycellium with Composition A showed
that both spores and mycellium were destroyed after 1
hour at room temperature. Composition A neutralised by
15 2%. TW80 was not effective however.

Example 4

20 Composition A was tested under the conditions
described in Example 2 for activity against poliovirus,
herpes simplex virus type 1 (HSV-1) and human
immunodeficiency virus type 1. 20 μ l of an appropriate
dilution of virus was mixed with 144 μ l of composition
A. After exposure, the virucide was neutralised by
25 addition of a 2% v/v solution of Tween 80 in lambda
buffer. In cell culture assays using HT-29 cells (for
poliovirus), MRC-5 cells for HSV-1 and IIUT-78 cells
for HIV-1 there was complete reduction of infectivity
of initial inocula of $1-4 \times 10^6$ virus. There was no
30 apparent cytopathic effect on MRC-5 cells or HT-29
cells.

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CLAIMS

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1. An antiviral or antifungal composition comprising an effective concentration of a mixture of a ferrous salt and an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers and maple leaves.

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2. A composition as claimed in claim 1, which is an aqueous solution.

3. A composition as claimed in claim 2, wherein the ferrous salt concentration is 0.1 mM to 0.1 M.

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4. A composition as claimed in claim 2 or claim 3, wherein the plant extract is used at a dilution of 1 to 100 times.

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5. A composition as claimed in any one of claims 2 to 4, wherein the iron salt is ferrous sulphate used at a concentration of 1 - 20 mM and the plant extract is pomegranate rind extract.

6. A solid or liquid concentrate which on dilution with water gives a composition according to any one of claims 2 to 6.

25

7. A method of controlling virus or fungus, which method comprises contacting the virus or fungus with an effective concentration of a ferrous salt and an effective concentration of an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves.

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8. A method as claimed in claim 7, wherein the ferrous salt is used as a 0.1 mM to 0.1 M aqueous solution.

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9. A method as claimed in claim 7 or claim 8, wherein the virus or fungus is on a surface and the ferrous salt and the plant extract are applied to the surface.

5 10. A method as claimed in any one of claims 7 to 9, wherein the virus or fungus is contacted with an aqueous composition comprising a mixture of the ferrous salt and the plant extract.

10 11. For use as an antiviral or antifungal agent, a composition containing a mixture of an effective concentration of a ferrous salt and an effective concentration of an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	DATABASE WPI Week 1878 Derwent Publications Ltd., London, GB; AN 78-32601A [18] & JP, A, 53 029 907 (HOTTA K) , 20 March 1978 see abstract	1-8
Y	DATABASE WPI Week 4791 Derwent Publications Ltd., London, GB; AN 91-346759 & US, A, 7 661 005 (US FOOD & DRUG ADMIN / US DEPT HEALTH & HUMAN SERVICES) , 15 October 1991 see abstract & WO, A, 94 04167 (...)	1-8

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A	DATABASE WPI Week 2490 Derwent Publications Ltd., London, GB; AN 90-181324 [24] & JP,A,02 117 608 (ITO-EN) , 2 May 1990 see abstract ---	1-8
A	DATABASE WPI Week 4889 Derwent Publications Ltd., London, GB; AN 89-353214 [48] & JP,A,01 265 023 (TAIYO CHEMICAL IND) , 23 October 1989 see abstract -----	1-8

INTERNATIONAL SEARCH REPORT

Interns Application No
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NONE